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PRESENT POSSIBILITIES FOR THE DETERMINATION OF INORGANIC ARSENIC AND ORGANOARSENICAL COMPOUNDS IN BIOLOGICAL FLUIDS

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Summary. - *A survey is presented of the applicability of atomic spectrometry and other techniques for the quantitation of both inorganic and organic arsenic in biological fluids. Pretreatment of samples and speciation of the different As forms are particularly emphasized. The potential of inductively-coupled plasma spectrometry combined with high performance liquid chromatography separations is also highlighted.*

Riassunto (Attuali possibilità per la determinazione dell'arsenico inorganico e dei composti organoarsenicali nei fluidi biologici). - *Viene presentata una panoramica sull'applicabilita della spettrometria atomica e di altre tecniche alla determinazione dell'As, sia organico che inorganico, nei fluidi biologici. Particolare enfasi è stata data al pretrattamento dei campioni e alla speciazione di differenti forme di As. Inoltre una certa attenzione viene posta alla combinazione della spettrometria atomica a plasma accoppiato induttivamente con tecniche ad elevato potere di separazione quali la cromatografia liquida ad alta risoluzione.*

Introduction

Atomic spectroscopy, be it emission or absorption, has played a fundamental role as an analytical tool for elucidating the metabolism of arsenic. In fact, these techniques possess the necessary requirements as regards specificity, sensitivity, accuracy and precision needed for the quantitation of this element at trace and ultratrace levels. Before the appearance of the now classical work of Braman and Foreback in 1973 [1], the analyst's interest had been restricted to the quantitative determination of "total arsenic" (TA). Nowadays, it is well known that TA present in biological fluids is the sum of arsenites: As(III); arsenates: As(V); monomethylarsonic acid: MMAA; dimethylarsinic acid: DMAA and others, deriving from the consumption of seafood (fish, shellfish and mussels). The need for separate determinations of the single arsenical forms is due to their different toxicological significance. The intake of inorganic arsenic (IA) by animal organisms, including man, implies a biotransformation that can be summarized as illustrated hereafter [2].

Inorganic As(III) or As(V) in humans are transformed mainly into DMAA and a considerably lower amount into MMAA for the 70-90% of urinary As. The liver seems to be the main side where the above process takes place. The said metabolites are much less toxic than inorganic As. Thus this biotransformation can be considered a detoxification that appears to be less effective when exposure is strong. It has been demonstrated that methylation is practically constant up to ingestion of *ca.* 500 µg/day for 5 days.

Therefore, analyses of TA do not differentiate between As of nutritional origin, of minor toxicological importance, and IA taken in through water, the working place and the environment in general, the latter being much more toxic. This does not allow the actual toxicity of the ingested dose to be evaluated. Moreover, if one considers that total arsenuria values can often be higher than 1 mg/l after intake of seafood, it is easily understood how wrong the conclusions could be.

Lauwerys *et al.* [3] reviewing the techniques used for the analysis of As(III), As(V), MMAA and DMAA in human urine stated that further investigation was needed to determine whether or not the As distribution found really represented the distribution of the above mentioned species secreted by the kidney. Buchet *et al.* [4] compared several methods for TA determination in water and urine, and for the study of As metabolism and monitoring of exposed workers. A method for sample preparation was the acidification of urine and extraction with toluene in presence or not of KI and finally mineralization with MgO a 600 °C. The same authors [5, 6] further studied the As metabolism after administration of single oral doses of arsenite, MMAA and DMAA and after repeated ingestion of Na metaarsenite. In both cases urinary excretion was measured by AAS after hydride formation. Regarding the first work it could be concluded that while DMAA is

excreted unchanged, 13% of MMAA is methylated into DMAA and 75% of the arsenic excreted after ingestion of arsenite is methylated to MMAA and DMAA in a 1:2 ratio. The latter study, of a more complex nature, allowed a conclusion to be reached: a steady state to the urinary excretion of As occurs, corresponding to about 60% of the ingested dose after five days.

While the above studies were made on humans, Vahter *et al.* [7] carried out a work on the metabolism of arsenobetaine in mice, rats and rabbits. These authors used an ^{73}As labelled arsenobetaine orally administered or intravenously injected and noted that elimination was, within 3 days, *ca.* 75% for rabbits and 98% for other animals. In mice, doses from 4 to 400 ng As/kg body weight did not influence the rate of excretion. Arsenobetaine was completely adsorbed along the gastrointestinal tract and not biotransformed. Arsenic was quantified by radioactivity measurements. The speciation of As(III) and As(V) in organic tissues after acid digestion (HNO_3, H_2SO_4) was made by Weigert *et al.* [8] who found that mineralization influenced the original valence in a not reproducible way.

Within this context, mention should be made herewith of the criteria developed by Zielhuis *et al.* [9] for the speciation of As in different media. The present survey aims at illustrating the most recent achievements in this research area.

Separation and analysis procedures

To date, reliable and accurate dry-ashing procedures are available for the determination of TA in biological fluids and other organic matrices. Among the most widely used, mention should be made of the one based on oxidation with MgO at 600 °C. All arsenical forms are thus converted into As(V) which can be determined as such or after reduction to As(III).

An alternative method based on hollow cathode emission spectrography for the quantitation of TA was described by Alimonti *et al.* [10]. Solutions obtained by wet ashing of biological materials containing As were put into steel hollow cathodes, dried under IR and calcined at 700 °C. Because of acidity, Ni was leached from cathodic material and reacted with As, thus forming nickel arsenide. This is a sparingly volatile compound consenting higher ashing temperatures to be reached without losing As.

In general terms, the quantitative determination of IA and organoarsenical should consist of two steps, namely: a) separation; b) quantitation of the individual species. Before going into a quick survey of the recent literature on this subject, it is worth recalling that insofar as point a) is concerned successfull results have been obtained by fractional distillation of arsine generated from the various arsenical forms, anionic and cationic chromatography, and HPLC with the use of ionic exchange and inverse phase columns. Regarding point b), mention should be made of flame, graphite furnace and heated quartz tube atomic absorption spectrometry (AAS) as well as of atomic emission spectroscopy, in particular with inductively-coupled plasma (ICP-AES).

Braman *et al.* [1, 11] were the first to carry out the simultaneous speciation of As(III), As(V), MMAA and DMAA in human urine and water samples. Their procedure involves the reduction of these arsenicals to arsine (A), methylarsine (MA) and dimethylarsine (DMA), condensation in a glass container dipped in liquid nitrogen, differential volatilization under He flow and subsequent detection of the three arsines by spark emission spectroscopy at 193.7 nm. This technique also permitted As(III) and As(V) to be differentiated simply by carrying out the reduction reaction at different pH values. Andreas [12] further refined this procedure by bubbling an inert gas in the sample to remove occasional pre-existing arsines. Detection of arsines originated by reduction of arsenicals is done by conveying the reduction gases to a hydrogen-air flame and reading absorbance at 193.7 nm after selective separation of arsines condensed at liquid nitrogen temperature. Alternatively, the gaseous mixture is conveyed to the chromatographic column (flame - ionization and electron - capture detection), bypassing the condensation tube.

Some authors have strived to clarify the importance the pH has on the medium where reduction is performed. Agget and Aspell [13] restricted their studies to IA suggesting the following reduction mechanism:

$$As(V) \longrightarrow As(III) \qquad (1)$$

$$As(III) \xrightarrow[\text{formation}]{\text{arsine}} AsH_3 \qquad (2)$$

Theoretical consideration of the redox potential of the As(V)/As(III) couple leads to the conclusion that at a 3.5 pH, reaction 1 is so slow that the formation of AsH_3 from As(V) is negligible compared to that of As(III) already present in the sample. The existence of a critical As(V)/As(III) ratio has been also ascertained. On this basis, the amount of AsH_3 originated from As(V) is no longer negligible when the said ratio exceeds 40.

Hinners [14] extended this type of study to MMAA and DMAA by defining the optimal pH value for the evaluation of arsines from such compounds. An interesting consideration made by this author is that while the conversion of MMAA in a buffer at pH 4.8 as compared to that of As(III) is only 12%, it becomes virtually complete if a reduction with KI-ascorbic acid is previously done. This suggests a reduction into methylarsenous acid $CH_3As(OH)_2$ or arsonomethane CH_3AsO. Arbab-Zavar and Howard [15] gave a detailed report of the formation curves for A, MA and DMA in a wide pH interval. The detection of arsines by atomic emission spectroscopy was at an early stage flanked to flameless atomic absorption spectrometry, both with a heated quartz tube and a graphite furnace.

This solution has been adopted for practical purposes, particularly owing to the great availability of commercial instruments.

The use of chromatographic techniques has gradually replaced separation procedures based on selective volatilization of the various arsines, thus rationalizing the approach to the problem of separation of arsenicals in diversified matrices. The work done by Tam *et al.* [16] is a milestone in this field. It is no wonder that the first separation of As(III), As(V), MMAA and DMAA in biological fluids was performed by means of a cationic resin. In fact, contrary to what their chemical nature would imply, MMAA and DMAA have a strong affinity for cationic resins. According to Tam's method their elution can be achieved simply with water and diluted ammonia. This behaviour, apparently anomalous, is explained by their amphoteric nature which allows the following equilibrium to be attained:

$$(CH_3)_2AsO_2H + H^+ \longrightarrow (CH_3)_2AsO_2H_2^+ \qquad (3)$$

Owing to the inductive effect of the two methyl groups, DMAA is more basic than MMAA. In Tam's paper, As(III) and As(V) are eluted simultaneously. On the other hand Dietz and Perez [17] have demonstrated that the two inorganic forms of arsenic can be completely separated on a column of adequate size. Separations exploit the fact that As(V) (pK of arsenic acid, 2.25) is not retained as it is entirely dissociated and that As(III) (pK of arsenious acid, 9.23) undergoes a process of physical retention. Recently, Vahter and Envall [18] have also demonstrated that As(III) at low levels can be partially oxydized during chromatographic separation on ion-exchange resins. Buratti *et al.* [19] employed Tam's procedure to separate metabolic forms of As in urine. The detection of these and of the TA, obtained after mineralization with MgO and $Mg(NO_3)_2$, was made by AAS.

The reduction of As compounds with sodium tetrahydroborate to obtain arsines after chromatographic separation and before determination by AAS was performed by Tye *et al.* [20], who made the preconcentration of samples with a laminar anion exchange column to improve the detection limits and remove matrix interferences. By using paired-ion, reversed phase HPLC followed by hydride generation, Bushee *et al.* [21] obtained better detection limits, a linearity response over a large concentration range and a good separation. In this case the detection of As was performed by ICP-AES. Nisamaneepong *et al.* [22] also used the reversed phase ion pair HPLC and ICP-AES for the speciation of As deriving from environmental pollution. The authors avoided reducing As compounds to arsines. Blood and urine of workers exposed to As_2O_3 were monitored by Foa *et al.* [23] with Tam's technique [16] for determining IA, MMAA and DMAA. TA was measured by AAS after ashing with the procedure using MgO at 600 °C described before.

HPLC with anion exchange column using a 15 mm linear gradient from water to 0.5 M $(NH_4)_2CO_3$ to separate arsenite, arsenate, MMAA and DMAA was developed by Spall *et al.* [24] to study the arsenic metabolism in cultured cell suspensions. The detection was accomplished by ICP-AES after direct coupling with the column. Low *et al.* [25] inserted a switching column between a C18 reversed phase and an anion-exchange column to separate As(III), As(V), MMAA, DMAA and arsenobetaine. The coupling with ICP-AES was made up to detect the element, thus reducing analysis time, creating sharper peaks and good detection limit. A kind of official acknowledgment was given by NIOSH [26] to the application of ion chromatography, hydride generation and AAS with graphite furnace for the analysis of organoarsenical compounds.

A drawback that could limit the accuracy of the procedures based on arsine evaluation is the possibility of a molecular rearrangement of the arsines themselves. For example, reduction of MMAA besides leading to MA can also lead to the formation of A, DMA and trimethylarsine (TMA). Moreover, the reduction of DMA forms small amounts of MA. Talmi and Bostick [27] optimized experimental parameters so as to minimize undesiderable side reactions and quantified arsenical forms after gas-chromatographic separation of arsine and detection by microwave emission spectroscopy. Analytical aspects related to the determination of IA, MMAA and DMAA by flameless atomic absorption spectrometry with graphite furnace have been faced by various authors. Among these, the contribution made by Chakrabarti *et al.* is worth mentioning [28]. They stated that low levels of As can be determined carefully only after the matrix has been separated. Significant interferences by Na, K and sulphate have been reported even at low amounts. Brinckman *et al.* [29] determined As(III), As(V), MMAA and DMAA in mixture after separation by HPLC on cationic and anionic resins and on reverse phase. Different detection powers have been found according to the various arsenical forms. The elution order on a phase-reversed column is As(III), DMAA, MMAA and As(V).

Stockton and Irgolic [30] applied a detection system based on flameless atomic absorption with Zeeman background correction to a mixture of As(III), As(V), arsenocholine and arsenobetaine after separation by HPLC on reverse-phase column. However, no detailed information was reported on quantitative data or on practical applications. An elegant example of separation of IA, MMAA and DMAA on single column [31] is based on the use of an anion exchange resin with low gradient elution ability. Contrary to what has been reported by other authors [29], under the adopted experimental conditions no different responses are found for the individual arsenical compounds detected by graphite-furnace AAS. No correction was claimed necessary for aspecific absorption.

An automatic system for the determination of As(III), As(V), DMAA, MMAA and *p*-aminophenylarsonic acid was described recently [32]. The procedure is based on separation with anionic column. The effluent is conveyed

on an apparatus for generating arsines, which, after gas-liquid separation, enters a heated quartz tube for the determination at 193.7 nm. Such methodology was studied for the analysis of synthetic mixtures, but it is foreseeable that it can be equally applied to various matrices, provided the role of other anions, capable of reducing the exchange ability of the resin, is clarified.

The literature on application possibilities of ICP-AES quantification of arsenical forms separated with chromatographic techniques, as described above, is to date relatively abundant. Subsequent to the first works on this topic is the paper by Barnes and Genna [33], providing a preliminary insight into this research area. Later Morita *et al.* [34] presented a procedure for HPLC separation of As compounds followed by ICP-AES detection. Chromatographic separations have been carried out on nucleosil-N-$(CH_3)_3$ and nucleosil-SO_3H-10 columns, anionic and cationic, respectively. While in the first case a complete separation is obtained, in the elution order of As (III), MMAA, DMAA, and As(V), in the second case As(III), As(V), MMAA and arsenobetaine are obtained. Selectivity of various elements has also been studied, with relatively low values for Al and Ti (70 and 33, respectively). Linearity is in the 50-1000 ng As range for each of the compounds studied. Odanaka *et al.* [35] approached the resolution of analytical problems in As speciation differently by combining gas-chromatography with a multiple ion detection (GC-MID) system and hydride generation-heptane cold trap (HG-HCT) technique. A and MA thus produced were collected in heptane at -80 °C and determined by GC-MID. Detection limit was about 0.2 mg/l. Materials analyzed included samples of soil, urine, river water and plant extracts. Recovery varied from 85 to 100%.

Conclusions

Although this survey does not claim to be complete the problem of speciation and quantitative determination of the various forms of arsenic can to date be faced with reliable and not excessively complex methodologies. On the other hand, biological monitoring poses serious analytical problems, which can greatly impair precision and accuracy of the results. Within this framework there are several aspects that deserve further analysis. First, there is a great deal of uncertainty and even inconsistency regarding sample storage procedures. Some authors reported [36] that no alterations in the distribution of different As metabolites or in TA concentration in urine samples can occur even after three months. However, the validity of these results is limited as the procedure followed did not provide separation of As(III) from As(V).

Second, the oxidation state of IA added to experimental animal feed and the one used for the preparation of analytical reference materials should be carefully controlled. In fact, not only do the two forms of IA show different toxicological properties, but also their detectability greatly differs when using analytical techniques such as flameless AAS with heated quartz tube. In this respect the procedure developed by Reay and Asher [37] is worth mentioning. By this procedure, all IA is preliminarly reduced to As(III) and then purified by ion-exchange chromatography. High-purity As(V) standards could thus be obtained *via* UV irradiation of the As(III)-containing solutions. Moreover it should be kept in mind that spontaneous transformation of As(III) into As(V), and *vice versa*, may take place as has been observed during ion-exchange chromatographic separation or separation on cationic resins [18]. In both cases, the oxidation reaction prevails even down to a concentration of 0.4 µg/l.

As outlined in this review, detection methods based on electrothermal AAS after chromatographic separation appear to be the method of choice. The vast majority of papers centered on this subject unfortunately report on the separation of synthetic aqueous solutions at concentrations well above those expected in biological fluids of non-exposed individuals. The quantification of the various forms of As by means of graphite furnace AAS also poses some limitations as regards detectability, generally of about 20 µg as absolute amount, corresponding to *ca.* 0.4 µg/l when injecting 50 µl volumes. Consequently, complex analytical procedures to account for aspecific absorption (e.g. Zeeman correction) are necessary since dilution of the samples cannot be made.

Finally, sufficiently high volumes must be used for quantification, as chromatographic elution inevitably causes sample dilution. Analysis with heated quartz tube technique offers this advantage, while not permitting more than 2-3 measurements for each chromatographic fraction as a rule. In its turn ICP-AES appears to have a high analytical potential for this kind of determination, although for the time being specific information is lacking. One main drawback of this technique is the not entirely adequate detection power it has for As, which can, however, be rather elegantly obviated by adopting the preconcentration procedure based on the use of poly(dithiocarbamate) resin suggested by Barnes and Genna [33]. The necessary manipulation of the sample is more than counterbalanced by the wide dynamic range and limited influence of matrix composition typical of the plasma spectrometry. Its combination with HPLC for real-time speciation and analysis is an additional promising feature, and it is thus all the more desirable that research in this field be further encouraged.

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